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### Authors

Reiner, SL  
Zheng, S  
Wang, ZE  
et al.

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# **Leishmania Promastigotes Evade Interleukin 12 (IL-12) Induction by Macrophages and Stimulate a Broad Range of Cytokines from CD4<sup>+</sup> T Cells during Initiation of Infection**

By Steven L. Reiner, Shichun Zheng, Zhi-En Wang,  
Linda Stowring, and Richard M. Locksley

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*From the Departments of Medicine and Microbiology/Immunology, University of California, San Francisco, San Francisco, California 94143*

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## **Summary**

*Leishmania major* are intramacrophage parasites whose eradication requires the induction of T helper 1 (Th1) effector cells capable of activating macrophages to a microbicidal state. Interleukin 12 (IL-12) has been recently identified as a macrophage-derived cytokine capable of mediating Th1 effector cell development, and of markedly enhancing interferon  $\gamma$  (IFN- $\gamma$ ) production by T cells and natural killer cells. Infection of macrophages in vitro by promastigotes of *L. major* caused no induction of IL-12 p40 transcripts, whereas stimulation using heat-killed *Listeria* or bacterial lipopolysaccharide induced readily detectable IL-12 mRNA. Using a competitor construct to quantitate a number of transcripts, a kinetic analysis of cytokine induction during the first few days of infection by *L. major* was performed. All strains of mice examined, including susceptible BALB/c and resistant C57BL/6, B10.D2, and C3H/HeN, had the appearance of a CD4<sup>+</sup> population in the draining lymph nodes that contained transcripts for IL-2, IL-4, and IFN- $\gamma$  (and in some cases, IL-10) that peaked 4 d after infection. In resistant mice, the transcripts for IL-2, IL-4, and IL-10 were subsequently downregulated, whereas in susceptible BALB/c mice, these transcripts were only slightly decreased, and IL-4 continued to be reexpressed at high levels. IL-12 transcripts were first detected in vivo by 7 d after infection, consistent with induction by intracellular amastigotes. Challenge of macrophages in vitro confirmed that amastigotes, in contrast to promastigotes, induced IL-12 p40 mRNA. Reexamination of the cytokine mRNA at 4 d revealed expression of IL-13 in all strains analyzed, suggesting that IL-2 and IL-13 may mediate the IL-12-independent production of IFN- $\gamma$  during the first days after infection. *Leishmania* have evolved to avoid inducing IL-12 from host macrophages during transmission from the insect vector, and cause a striking induction of mRNAs for IL-2, IL-4, IL-10, and IL-13 in CD4<sup>+</sup> T cells. Each of these activities may favor survival of the organism.

**T**he resistance or susceptibility of inbred strains of mice to *Leishmania major* correlates with the induction of parasite-specific Th1 or Th2 responses, respectively (1–4). Susceptibility is not related to the absence of precursors capable of parasite-specific Th1 development, however, since a number of immunologic interventions enables such mice to heal and develop protective Th1 cells (5–9). Such interventions must be given within the first week of infection in order that redirection of the CD4 subset response can be accomplished; interventions at later times are unsuccessful. These studies suggest a critical early period after infection in which Th1 or Th2 effector subsets become established.

Although several experimental systems suggest a role for the type of APCs (10) or MHC class II haplotypes (11) in the developmental regulation of Th1 or Th2 cells, recent data

using limiting dilution analysis (12) and transgenic TCR mice (13, 14) suggest that cytokines themselves may be the most significant determinants in the maturation of CD4<sup>+</sup> precursors to Th1 or Th2 phenotypes. An extensive analysis of CD4<sup>+</sup> T cells responding during the course of *L. major* infection was consistent with this model. Immunodominant CD4<sup>+</sup> cells of both the Th1 and Th2 phenotype used comparable TCRs, consistent with their evolution from common precursors (15). This analysis suggests that the cytokine milieu that occurs during the initial 1–2 wk after infection plays a major role in shaping the subsequent development of protective Th1 or noncurative Th2 cells. In vitro studies have identified a potentially important role for IL-12 (16, 17) in mediating Th1 cell development. Indeed, treatment of susceptible BALB/c mice with recombinant IL-12 mediates cure

in these animals associated with the production of Th1-like cytokines (18, 19), and neutralization of IL-12 leads to an adverse outcome in resistant mice (19). This effect may be mediated through induction of IFN- $\gamma$  (18), and can be negatively regulated by IL-10 (13). Although neutralization of IFN- $\gamma$  impedes the development of cure (8, 20), IFN- $\gamma$  itself is not capable of mediating sustained Th1 cell development in susceptible mice (8, 21). IL-4 has been implicated in Th2 development (13, 14, 16, 22–24) and inhibition of Th1 development (25) in *in vitro* studies. Neutralization of IL-4 in infected BALB/2 mice induces curative Th1 cell development (8). IL-2 is required for IL-4 production (24, 26), and neutralization of IL-2 was also capable of reversing susceptibility in the *L. major* model (27). It should be noted that these responses are specific to *L. major*; infection of BALB/c mice with *Candida albicans* leads to the development of protective Th1 responses (28).

Based on these considerations, we undertook a detailed analysis of the cytokine response after the interaction of *L. major* with its host cell, the macrophage, *in vitro*, and during the early periods after infection *in vivo*. Rather than rely on restimulation of bulk lymphocytes *in vitro* (29) or analysis of limiting dilution assays (30, 31), we have employed a system of competitive quantitative reverse transcriptase-PCR (RT-PCR) (32) to analyze the cytokine response in defined cell populations after parasite challenge. This method has allowed us to identify several novel features of the immunopathogenesis of murine leishmaniasis and to delineate the critical intervals during which the Th1/Th2 switch occurs in this model.

## Materials and Methods

**Animals and Parasites.** 6–8-wk-old BALB/c, C57BL/6, B10.D2, C3H/HeN, and C.B-17/*scid* (SCID) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the University of California, San Francisco (UCSF) animal facility. Infections were performed with promastigotes of *L. major* (strain WHOM/IR/-/173) grown in M199 media (UCSF Cell Culture Facility) supplemented with 30% fetal bovine serum and antibiotics. Metacyclic organisms were isolated by negative selection after incubation of stationary-phase cultures with peanut lectin-coated beads (Sigma Chemical Co., St. Louis, MO) as described (33). In designated experiments, log-phase procyclic organisms were collected after 48 h of culture. Animal experiments used only metacyclics, which represent the mature infectious stage of the promastigotes (34, 35). Animals were injected with  $5 \times 10^5$  organisms into each hind footpad in groups of three-to-five mice for each experimental point. Groups of coinfecting BALB/c mice were maintained beyond the early time points of analysis to establish the virulence of the inocula. Control groups of animals received sham injections using the same volume of balanced salt solution. Designated mice were treated with mAb GK1.5 (anti-CD4) at the time of infection as described (7). Amastigotes were purified after disruption of footpad tissues from 6-wk-infected BALB/c mice and centrifugation through a Percoll gradient (Pharmacia, Inc., Piscataway, NJ) as described (7).

**Macrophages and Cell Lines.** Bone marrow macrophages were established as described (36). Briefly, femurs from BALB/c and C57BL/6 mice were isolated and the marrow evacuated using a 1-ml tuberculin syringe and 27-gauge needle filled with balanced salt solution. Cells were grown in complete DME H21 (UCSF

Cell Culture Facility) containing L cell-conditioned media (30% vol/vol), antibiotics, and 10% FCS (Hyclone Laboratories, Logan, UT) for 1 wk until uniform monolayers of macrophages were established. After washing, stimuli were added at the following concentrations: metacyclic or procyclic *L. major*, 2–20 organisms per macrophage; amastigotes, 1–10 organisms per macrophage; LPS (*Escherichia coli* 055:B5; Sigma Chemical Co.), 50 ng/ml; heat-killed *Listeria monocytogenes* (HKLM)<sup>1</sup>,  $10^6$  CFU equivalents/ml. After 6 or 24 h, monolayers were washed with warm balanced salt solution, and the cells were removed from the wells by flushing with RNazol (Biotech, Houston, TX).

**Collection of Cells and Tissue.** At designated periods after infection, the popliteal LN and footpads were flash frozen in liquid nitrogen, pulverized to a fine powder, and placed in RNazol. For analysis of cell subsets, popliteal LN were excised, teased to single cell suspensions, and incubated with designated mAbs against CD4<sup>+</sup> (GK1.5; Becton-Dickinson & Co., Mountain View, CA), CD8<sup>+</sup> (YTS 169.4; Caltag Laboratories, San Francisco, CA), or NK1.1 (PK 136; Pharmingen, San Diego, CA). Cells were selected in a magnetic field using the appropriate secondary mAbs coupled to ferrous beads (Advanced Magnetics, Inc., Cambridge, MA) as described (2). Isolations were carried out entirely at 4°C. The purity of the designated selections exceeded 90% in all groups as assessed by FACS<sup>®</sup> analysis (Becton Dickinson & Co.) in select experiments. Selection by this method resulted in no induction of cytokine transcripts in resting lymphocyte populations.

**Competitive Quantitative RT-PCR.** Total RNA was extracted using RNazol according to the manufacturer's directions. Specimens were quantitated both by optical density readings and intensity of ethidium staining on agarose gels. Reverse transcription was performed using murine Moloney leukemia virus RT (GIBCO BRL, Bethesda, MD) and random hexamer primers (Promega, Madison, WI) as described (15, 32). PCR was performed using a multiple cytokine-containing competitive construct as described in detail elsewhere (32). Briefly, aliquots of cDNA were first assayed for levels of the constitutively expressed gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), by placing equal concentrations of the competitor construct in each reaction and examining the ratio of competitor-to-wild-type band intensity after amplification with HPRT-specific primers. At the number of cycles performed, this visual inspection could discern differences as small as 1.25-fold (32). Adjustments were made in the amount of experimental cDNAs needed to standardize the HPRT levels to comparable levels among all groups. These adjusted volumes of cDNA were then used to quantitate cytokine levels using a fixed concentration of competitor in each reaction in the presence of the cytokine-specific primers. The primers used for amplification were as described (32) with the addition of the following: macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ): 5'-ATGAAGGTCTCCACCACTGCCCTTG; 3'-GGCATTTCAGTTCAGGTCAGTGAT; IL-13: 5'-CTTGCTTGCCTTGGTGGTCTCGC; 3'-GCAGTTTGTATAAAGTGGGGCT. Cytokine analyses were repeated and the HPRT levels were reestablished at the end of each experiment to confirm their reproducibility. In this assay, separation of the amplification products after electrophoresis on a 2.5% ethidium-stained agarose gel allows the discrimination of the larger competitor construct from the unknown, wild-type cDNAs that migrate farther in the gel. For most experiments, the differences in band intensities between the wild-type and competitor amplifications between groups allowed

<sup>1</sup> Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; HKLM, heat-killed *Listeria monocytogenes*; HPRT, hypoxanthine-guanine phosphoribosyl-transferase; MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$ .

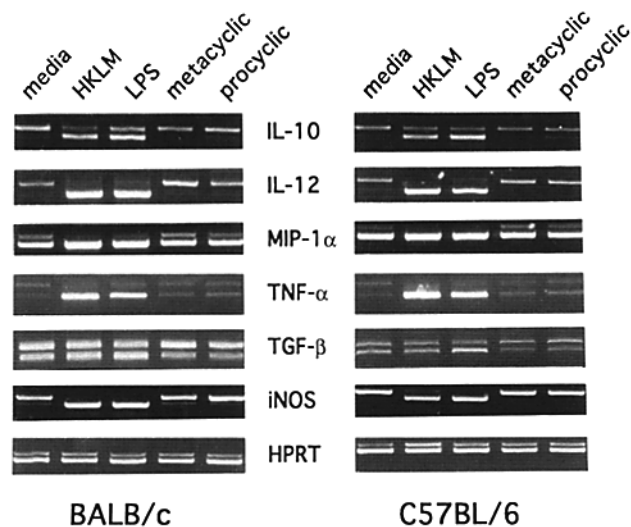
ready qualitative discrimination of the relative mRNA levels. In selected experiments, including the kinetic analyses, precise quantitation was achieved using standard titrations of the competitor against a constant cDNA input and used to normalize transcript levels to expression in resting tissues (32).

## Results

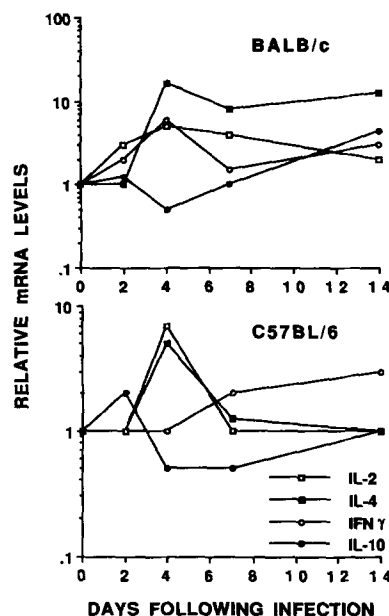
***Leishmania major* Promastigotes Evade Induction of Macrophage Cytokines In Vitro** *Leishmania* invade and replicate only within macrophages in the host. Macrophages can serve as the source of cytokines such as IL-10 and IL-12 that modulate the development of CD4<sup>+</sup> subsets (13, 16). Bone marrow macrophages from BALB/c and C57BL/6 mice were isolated and incubated for 24 h with metacyclic or procyclic promastigotes of *L. major*, heat-killed *Listeria* or LPS (Fig. 1). Both HKLM and LPS were potent inducers of mRNA for IL-10, IL-12 (as assessed by upregulation of p40 subunit expression [37, 38]), MIP-1 $\alpha$ , TNF- $\alpha$ , and inducible nitric oxide synthase (iNOS). In contrast, neither the metacyclic nor procyclic forms of *L. major* induced any of these mRNA from macrophages under these conditions, with the exception of small but consistent increases in TNF- $\alpha$ . None of these stimuli induced the appearance of TGF- $\beta$  transcripts significantly above the resting level. Variation of the inocula of promastigotes such

that 30–90% of macrophages were infected with multiple amastigote forms at the conclusion of the assay or earlier analysis at 6 h after infection resulted in no differences in this pattern of cytokine transcription (data not shown). Similar results were obtained using the P388.D1 and J774 macrophage cell lines (data not shown).

**Induction of Cytokine Gene Transcription by *Leishmania* In Vivo** Susceptible BALB/c and resistant C57BL/6 mice were infected in the hind footpads with metacyclic *L. major* promastigotes and killed immediately or 2, 4, 7, or 14 d later. Analysis of cytokine mRNA levels in whole LN by quantitative RT-PCR revealed a highly stereotyped pattern (Fig. 2); no cytokines were induced at these periods after infection with balanced salt solution. Both strains of mice exhibited a burst of IL-2 and IL-4 mRNA between 2 and 4 days after infection. Peak levels of IL-4 mRNA were usually higher in BALB/c mice (up to 16-fold) than in C57BL/6 (up to eight-fold), but were comparable in some experiments at 4 d. By 7 d, there was a marked decline in IL-2 and IL-4 mRNA in both groups of mice, although the decline in IL-4 was consistently less in BALB/c. By 14 d, IL-4 mRNA levels were reestablished at high levels in the BALB/c mice, whereas expression essentially returned to baseline levels in C57BL/6 animals. IL-2 mRNA closely followed IL-4 mRNA levels in C57BL/6 mice.



**Figure 1.** Induction of cytokine mRNA by *Leishmania* in host macrophages. BALB/c and C57BL/6 bone marrow-derived macrophages were incubated for 24 h in media alone, or challenged with heat-killed *Listeria* (HKLM), bacterial lipopolysaccharide (LPS), or metacyclic or procyclic promastigotes of *L. major*. Monolayers were washed and the RNA purified and used for RT-PCR. The input cDNAs were adjusted to yield comparable ratios of the competitor (upper band in each reaction) to wild-type (lower band in each reaction) intensities in the amplification reaction for the constitutively expressed product, HPRT (bottom), as resolved on a 2.5% ethidium-stained agarose gel. These input cDNAs were used in subsequent RT-PCR reactions using the designated oligomers for cytokines or inducible nitric oxide synthase (iNOS). IL-12 transcripts represent the inducible p40 component of IL-12. HPRT levels were assayed again at the conclusion of the experiment to establish the reliability of the cDNA volumes. Results are representative of five comparable experiments.

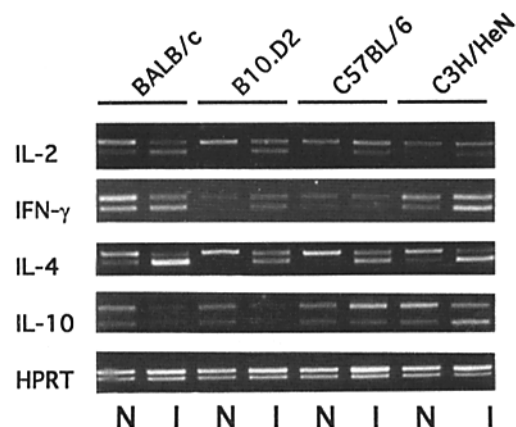


**Figure 2.** Cytokine mRNA in the draining LN after infection with metacyclic *L. major*. BALB/c (top) and C57BL/6 (bottom) mice were killed at designated time points after infection and the draining LN mRNA levels for the designated cytokines were assayed using RT-PCR. The kinetics of transcriptional changes for each gene were expressed relative to the levels expressed in the LN of uninfected animals, that were assigned a value of one. The cDNA concentrations examined at each time point were standardized by equalizing the HPRT mRNA levels. Note the vertical scale in the BALB/c group extends one order of magnitude higher than the C57BL/6 group. The results represent one of two comparable experiments. Variations between replicates within each experiment were <10%.

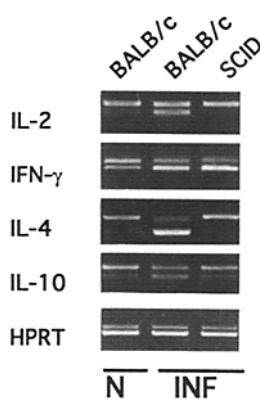
Levels of IFN- $\gamma$  rose steadily in both groups, but the BALB/c mice consistently maintained an earlier rise as compared to a late sustained increase in resistant C57BL/6 mice. C57BL/6 mice had negligible increases in IFN- $\gamma$  mRNA until 7 d after infection, coincident with the decrease in IL-4 mRNA. BALB/c mice displayed a steady incremental rise in IL-10 mRNA from days 4 through 14, whereas resistant C57BL/6 mice had reproducible downregulation of IL-10 transcripts between 4 and 7 d, followed by a slow return to baseline, uninfected levels.

**The IL-4 Burst at 4 d Is Characteristic of the Early Immune Response to *L. major*.** To determine whether other resistant strains of mice exhibited the same induction of cytokines at 4 d after infection, cytokine mRNA expression in infected BALB/c mice was compared to expression in infected C57BL/6, C3H/HeN, and B10.D2 strains (Fig. 3). All infected animals displayed a burst of IL-4 and IL-2 mRNA expression at 4 d, with at least fivefold elevations in all resistant strains tested. IFN- $\gamma$  mRNA levels were more variable, with highest levels in BALB/c and C3H/HeN mice, and lowest levels in C57BL/6 animals.

**Origin of IL-2 and IL-4 mRNA in CD4<sup>+</sup> Cells.** To assess the contribution by T cells to these cytokine transcripts, SCID mice were infected with *L. major* and the draining LN populations harvested at 4 d and used to examine cytokine mRNA levels (Fig. 4). BALB/c mice, congenic with SCID except for a portion of chromosome 12 (39), were analyzed concurrently. As compared to infected BALB/c mice, SCID mice expressed no detectable transcripts for IL-2 or IL-4, had markedly decreased levels of IL-10 mRNA, and demonstrated slightly enhanced levels of IFN- $\gamma$  mRNA. These data were consistent with the interpretation that IL-2 and IL-4 were derived from T cells, IL-10 was derived primarily from T cells, and IFN- $\gamma$  could be derived from a non-T cell compartment, presumably NK cells, previously demonstrated to be an important source of IFN- $\gamma$  in this disease (40).



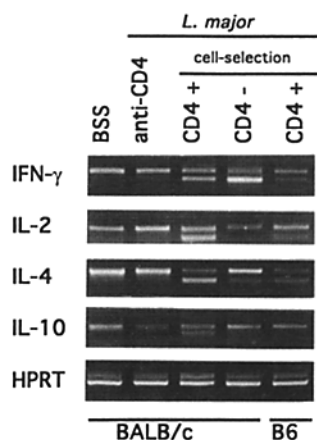
**Figure 3.** Cytokine mRNA in susceptible and resistant mice. Popliteal LN from groups of three from naive (N) and infected (I) animals of the designated strains were harvested 4 d after infection. Competitive PCR was performed for the designated cytokines after standardizing the cDNA concentration to the HPRT amplification signal.



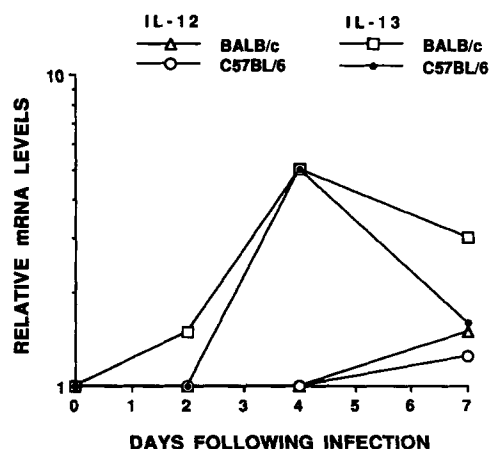
**Figure 4.** Cytokine mRNA in SCID mice infected with *L. major*. Groups of three BALB/c or congenic SCID mice were infected (INF) with metacyclic *L. major* and the LN harvested after 4 d. After purification of RNA, the designated cytokine mRNAs were compared by adjusting for comparable HPRT input, and compared to uninfected BALB/c (N). Resting levels of these mRNAs in SCID mice were comparable to the uninfected BALB/c group.

To further delineate the T cell lineage for these cytokine transcripts, BALB/c mice were depleted of CD4<sup>+</sup> T cells at the time of infection by treatment with mAb GK1.5 before harvesting the LN cells after 4 d of infection (Fig. 5). Treatment with anti-CD4 abolished cytokine transcripts for IL-2, IL-4, IL-10, and IFN- $\gamma$ , suggesting that CD4 T cells are required for the expression of these mRNAs. To quantitate cytokine mRNA expression in CD4<sup>+</sup> T cells directly, these cells were purified from infected BALB/c and C57BL/6 mice using magnetic beads before purification of mRNA and RT-PCR (Fig. 5). By this assay, CD4<sup>+</sup> cells expressed all of these cytokine transcripts in both strains of mice, and were the major source of IL-2, IL-4, and IL-10. Although CD4<sup>+</sup> T cells contributed mRNA for IFN- $\gamma$ , the major source of these transcripts occurred in the CD4<sup>-</sup> population of LN cells. This population was identified in infected C57BL/6 mice as cells expressing the NK1.1 marker by magnetic bead selection of CD4<sup>+</sup>, CD8<sup>+</sup>, and NK1.1<sup>+</sup> cells before mRNA purification and RT-PCR for IFN- $\gamma$ . By this analysis, IFN- $\gamma$  expression was greatest in NK1.1<sup>+</sup> cells, less in CD4<sup>+</sup> cells, and least in CD8<sup>+</sup> cells (data not shown).

By 14 d after infection, purification of CD4<sup>+</sup> T cells revealed substantial polarization of cytokine transcripts between susceptible BALB/c and resistant C57BL/6 mice, with elevated IL-4 and IL-10 consistently present in BALB/c cells and elevated IFN- $\gamma$  present in C57BL/6 cells (15, and data not shown).



**Figure 5.** Cellular sources of the early cytokine burst in murine leishmaniasis. Groups of five mice injected with *L. major* or balanced salt solution (BSS) were assayed for mRNA for the designated cytokines 4 d after inoculation. The infected animals were divided into those pretreated with anti-CD4 mAb before harvest of the LN, and those whose LN cells were selected into CD4<sup>+</sup> (BALB/c or C57BL/6 [B6]) or CD4<sup>-</sup> (BALB/c) populations using mAbs coupled to magnetic beads before mRNA extraction.

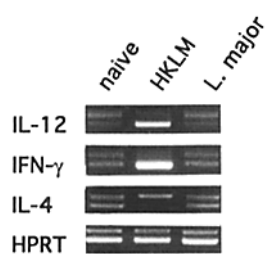


**Figure 6.** Induction of IL-12 p40 and IL-13 mRNA after infection with *L. major*. Groups of five BALB/c or C57BL/6 mice were inoculated with metacyclic *L. major* and the LN harvested at the designated periods after infection. After isolation of mRNA, transcripts were analyzed for expression of IL-12 p40 and IL-13 mRNA using RT-PCR. Results were comparable in three separate experiments.

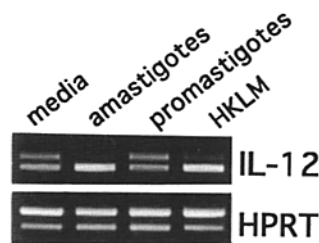
In contrast to their distribution at 4 d, the IFN- $\gamma$  transcripts were contained predominantly in the CD4<sup>+</sup> and not the CD4<sup>-</sup> compartment.

Footpads were harvested at 2 and 4 d after infection to assess whether significant amounts of these cytokines could be detected at earlier periods at the site of parasite inoculation. None of these transcripts were reliably detected at these early periods in tissue, and IL-4 mRNA did not appear at the footpad site until day 7 or later after infection in both resistant and susceptible mice (data not shown).

**Delayed Induction of IL-12 In Vivo Correlates with the Appearance of Amastigotes, which Induce IL-12 in Macrophages.** Investigations of induction of IL-12 p40 transcripts in vivo were carried out in BALB/c, C57BL/6 and C3H/HeN mice. None had detectable induction of IL-12 p40 mRNA at day 2 or 4 after infection in 12 separate experiments. A typical experiment in BALB/c and C57BL/6 mice is shown (Fig. 6). Transcripts for IL-12 p40 first appeared between 7 and 14 d after infection, and were typically 10-fold higher in BALB/c as compared to C57BL/6 mice (data not shown). To test the sensitivity of the in vivo assay for detecting IL-12 p40 mRNA induction, BALB/c mice were either left untreated, or injected with either HKLM or *L. major* metacyclic promastigotes (Fig. 7). Under these conditions, HKLM caused striking in-



**Figure 7.** Inoculation of *Leishmania* or heat-killed *Listeria*. Groups of three BALB/c mice were left uninfected (naive), or were inoculated in the hind footpads with 10<sup>7</sup> CFU heat-killed *Listeria monocytogenes* (HKLM) or metacyclic *L. major*, killed after 2 d and the popliteal LN cells examined for levels of IL-12 p40, IFN- $\gamma$  and IL-4 mRNA using RT-PCR standardized to HPRT levels.

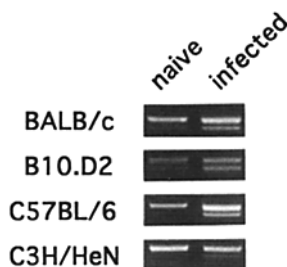


**Figure 8.** Amastigotes of *L. major* induce IL-12 p40 mRNA. Bone marrow-derived macrophages were incubated for 24 h in media alone or with a 10:1 ratio of *L. major* amastigotes or metacyclic promastigotes, or with heat-killed *Listeria* (HKLM). RNA was purified and analyzed for induction of IL-12 p40 mRNA as detailed in the legend to Fig. 1. Results represent one of three comparable experiments.

duction of IL-12 p40 and IFN- $\gamma$  mRNA, and, in contrast to *L. major*, suppressed transcripts for IL-4 below resting levels.

The delayed appearance of IL-12 transcripts in vivo, together with prior reports demonstrating the capacity of antibodies to IL-12 to affect adversely the outcome of infection with *L. major* (19), suggested that tissue amastigotes might be responsible for inducing IL-12 p40 mRNA. Amastigotes were purified from infected footpad tissues and used to inoculate bone marrow-derived macrophages in vitro. In contrast to promastigotes, amastigotes induced IL-12 p40 transcripts in a manner comparable to HKLM (Fig. 8). Further, incubation of amastigotes, but not promastigotes, with SCID splenocytes induced the dose-dependent release of IFN- $\gamma$  (Reiner, S. L., and R. M. Locksley, unpublished observations), consistent with IL-12 protein release (41).

IL-12 has been identified as a major stimulus for IFN- $\gamma$  production from NK cells (41–43). The absence of IL-12 induction by promastigotes raised questions regarding the stimulus for the induction of IFN- $\gamma$  in the NK population before day 7 (40). IL-13 is a recently described cytokine that, in human systems, has been demonstrated to induce the production of IFN- $\gamma$  from NK cells both alone and synergistically with IL-2 (44). LN were isolated 4 d after infection from BALB/c, B10.D2, C57BL/6, and C3H/HeN mice, and examined for IL-13 transcripts using RT-PCR (Fig. 9). In each of these mice, IL-13 mRNA could be readily detected; peak expression occurred at 4 d (Fig. 6). Examination of transcripts using mRNA purified from the CD4<sup>+</sup> and CD4<sup>-</sup> cell populations in both infected BALB/c and C57BL/6 mice confirmed expression of IL-13 mRNA in the CD4<sup>+</sup> population at 4 d (data not shown).



**Figure 9.** IL-13 mRNA is induced 4 d after infection with *L. major*. Groups of five mice of the designated strains were either left uninfected (naive) or infected with metacyclic *L. major* and the popliteal LN harvested at 4 d for analysis of IL-13 mRNA expression using RT-PCR standardized to HPRT (data not shown).

## Discussion

*Leishmania* are intramacrophage parasites whose survival requires evasion of CD4-mediated activation of macrophages to a microbicidal state. The major cytokine produced by CD4<sup>+</sup> T cells mediating macrophage activation against *Leishmania* is IFN- $\gamma$ , and neutralization of IFN- $\gamma$  renders otherwise resistant mice unable to restrict the progression of disease (20). Recently, IL-12 has been implicated in mediating IFN- $\gamma$  production by both T cells and NK cells (43, 45) and has been demonstrated to play a major role in the development of Th1 effector CD4 cells (16, 17). As demonstrated here, *Leishmania* promastigotes evade induction of IL-12 by macrophages in vitro and during infection in vivo, suggesting that this organism has evolved to enter macrophages using pathways that preclude the host's use of this important IFN- $\gamma$ -inducing pathway. Additional cytokines produced by macrophages, including MIP-1 $\alpha$  and IL-10, were similarly not induced by this organism, and TNF- $\alpha$  was only minimally induced. Our inability to document the induction of transcripts for TGF- $\beta$  suggests that the observed enhancement in TGF- $\beta$  production reported by others (46) may reflect post-translational regulation. Metacyclic promastigotes of *L. major* enter macrophages primarily using the CR1 (CD35) receptor (47), suggesting that cross-linking of CR1 may not effectively trigger macrophage cytokine production. This is in contrast to organisms such as *Listeria* or *Toxoplasma* (which use laminin-binding integrin receptors [48]), and the LPS of gram-negative organisms (which binds to CD14 [49] or CR3 [50]), all of which induce IL-12 production from macrophages in vitro and in vivo (16, 45, 51). As demonstrated here, one consequence of the relatively "silent" entry utilized by *Leishmania* was the failure to transcribe iNOS, an indispensable component of the priming of macrophages to an antileishmanial state (52, 53).

Although recombinant IL-12 has been demonstrated to enable susceptible BALB/c mice to heal *L. major* infection (18, 19), the data presented here suggest that failure to activate IL-12 transcription in vivo does not underlie the genetic susceptibility to *L. major*. IL-12 transcripts were first detected in all strains only seven or more days after infection. Importantly, in resistant strains of mice, generation of IL-12 occurred at a time after IL-4 and IL-10 became downregulated, thus leaving IFN- $\gamma$  unimpeded in the activation of macrophages. In contrast, susceptible BALB/c mice maintained significant levels of IL-4 and IL-10 during the period of IL-12 induction, suggesting that IFN- $\gamma$  produced in this period may be incapable of activating macrophages (54–57) or that the effects of IL-12 may be significantly attenuated (13). The capacity of IL-12 to cure BALB/c mice (18, 19) and the failure of IFN- $\gamma$  to cure (8), suggests that some of the effects of IL-12 may be IFN- $\gamma$  independent. Similar conclusions have been generated in human systems of IL-4-mediated Ig class switching (58).

The observation that polyclonal antisera against IL-12 were capable of abrogating control of *L. major* in resistant C57BL/6 (19) suggests that IL-12 induced after 7 d is required for maximizing the host control of the parasite. In contrast to the

promastigotes naturally inoculated by the sandfly vector, the tissue amastigotes induced readily detectable transcripts for IL-12 p40 when incubated with macrophages in vitro, and the appearance of IL-12 transcripts after infection in vivo correlated with the period during which amastigotes rupture and spread from infected macrophages. The mechanisms by which amastigotes, as opposed to promastigotes, induce IL-12 remains unknown, although preliminary experiments suggest that the lipophosphoglycan surface antigen, LPG, of promastigotes inhibits the induction of IL-12 from stimulated macrophages (Reiner, S. L., and R. M. Locksley, unpublished observations). Promastigotes of *Leishmania donovani* were significantly more susceptible to macrophage-mediated microbicidal activities than were amastigotes (59), suggesting that once transformation occurs, amastigotes are more prepared for intracellular survival. Indeed, persistence of *L. major* amastigotes for prolonged periods has been documented, even in resistant strains of mice (60, 61).

In contrast to their relatively silent invasion of macrophages in vitro, *Leishmania* promastigotes induced broad, Th0-like (62) cytokine expression in CD4<sup>+</sup> T cells that peaked 4 d after infection. This was true in all strains of mice examined and occurred irrespective of healer or nonhealer phenotype. Although the patterns of cytokine induction were similar in these mice in over 15 experiments, quantitative differences between strains were consistently noted. This was particularly true for IFN- $\gamma$  mRNA, for which C3H/HeN and BALB/c mice were consistently high producers, whereas C57BL/6 mice expressed low levels of this cytokine. These quantitative differences did not correlate with outcome of infection, however, suggesting that expression of IFN- $\gamma$  mRNA at early periods after infection does not correlate with the healer or nonhealer phenotype. Differences in IFN- $\gamma$  production presumably reflect differences in the NK compartments between these strains of mice (40).

The demonstration of induction of a reproducible Th0 phenotype at 4 d is consistent with the development of Th1 and Th2 cells from a common precursor, as suggested by studies of TCR usage in *L. major* infection (15) and in modulation of CD4 subset development in cells from TCR transgenic mice (13, 14). Earlier studies using different methods corroborate these findings. Thus two laboratories have established the capacity to recover CD4<sup>+</sup> T cells that produce both IL-4 and IFN- $\gamma$  within the first week after infection with *L. major* from both susceptible and resistant strains (30, 31). These investigators used restimulation of isolated LN cells followed by limiting dilution analysis to recover secreted cytokines from supernatants generated in vitro, and the hypothesis was advanced that the failure of BALB/c mice to cure correlated with a failure to downregulate the secretion of IL-4 (31). We have generated similar data using a transcriptional approach that avoids potential artifacts induced during restimulation in vitro when cells may be isolated from the natural milieu. In contrast, our approach does not document the efficient translation and secretion of these cytokines. In agreement with the earlier studies, stimulation of lym-

phocytes isolated 4 d after infection of both susceptible and resistant mice with the mitogen Con A yielded detectable IL-4 and IFN- $\gamma$  by both ELISA and ELISPOT assays in both susceptible and resistant mice (Reiner, S. L., unpublished results). The cytokines expressed by T cells are regulated almost entirely at the transcriptional level (63), and the presence of sequences in the 3'-untranslated regions of these mRNAs confers short half-lives on these pleiotropic mediators of the immune response (64). IL-12 is unusual among these cytokines in consisting of a heterodimer. The p35 subunit is constitutively expressed in a number of cell types, including macrophages (51), but is not efficiently secreted in the absence of the p40 subunit (37). The latter is transcriptionally induced in macrophages by a number of stimuli and contains the typical 3'-untranslated cytokine sequences conferring short mRNA half-life (38, 65). No instances in which induction of p40 mRNA in macrophages has not resulted in secretion of bioactive p35/p40 IL-12 have been described (45). The corroboration of much of the findings using protein-based assays by the transcriptional approach used here suggests the validity of this experimental strategy, which has the advantage of examining increased numbers of cytokines due to the powers of a PCR-based approach. At the same time, some differences are apparent in these studies. Whereas Morris et al. (30) could detect IL-4 from lymphocytes from C57BL/6 mice at 14 d after infection, we could detect little IL-4 mRNA in vivo during this period. Thus, some differences due to the experimental methodologies, the site of inoculation (footpads in this study versus base of the tail in the earlier study), or our use of selected metacyclic organisms, were observed.

The most striking differences between susceptible and resistant mice in this analysis was the failure of susceptible BALB/c mice to downregulate transcripts for IL-4 after their induction 4 d after infection. In contrast, resistant mice downregulated IL-4 transcripts to baseline, uninfected, levels by 7–14 d after infection. Such downregulation left IFN- $\gamma$  mRNA unopposed in resistant mice. These data suggest that a number of cytokine transcripts (IL-2, IL-4, and IL-10), with the notable exclusion of IFN- $\gamma$ , have the capacity to be coordinately downregulated after their induction, and further suggest that BALB/c mice may have a defect in this regulatory pathway. Alternatively, BALB/c mice may appropriately downregulate cytokine transcripts in an early Th0 population of cells that is overtaken by an expanding population of Th2 cells between days 2 and 7.

Two other features of the host response to *L. major* were noteworthy. First, CD4<sup>+</sup> T cells were the only source of IL-2 and IL-4, as assessed in studies of both SCID mice and CD4-depleted animals. Despite similar analysis of footpad tissues after infection, we were unable to document the early appearance of IL-4 at this site. This suggests that, as in influenza infection (66), the initial cytokine transcripts ap-

pear in the draining regional LN and then subsequently are apparent in tissues as sensitized T cells recirculate to the primary site of antigenic inoculation. It is still possible that preformed IL-4, as may occur in human mast cells (67), may be released in tissues and contribute to the initial cytokine burst in the draining LN. Further study will be required to address this possibility. Second, CD4<sup>+</sup> T cells were required for the induction of IFN- $\gamma$  transcripts, despite the observation that the major source of IFN- $\gamma$  mRNA was in the NK population. The evasion of IL-12 induction by promastigotes of *Leishmania* suggested alternative, T cell-dependent, pathways for IFN- $\gamma$  production, as demonstrated by the disappearance of these transcripts after administration of anti-CD4 antibody. Studies using human peripheral blood cells stimulated with anti-CD3 and PHA have demonstrated that ~50% of the IFN- $\gamma$  produced is due to the endogenous production of IL-12 (45), thus confirming an IL-12-independent, T cell-dependent pathway for IFN- $\gamma$  production. The capacity of IL-13 to synergistically enhance IFN- $\gamma$  production from NK cells (44), and the demonstration of IL-13 in Th0 clones (68), suggested that this cytokine may contribute to the induction of IFN- $\gamma$  transcripts in the NK population, perhaps in conjunction with IL-2 (44), shortly after infection with *L. major*. IL-13 mRNA could be readily detected in the CD4<sup>+</sup> T cell population 4 d after infection in all strains of mice examined. Aside from its role in enhancing NK-derived IFN- $\gamma$ , IL-13 most closely resembles IL-4 in its activities (44, 68, 69), presumably because of its capacity to share components of the IL-4 receptor (70). Further evaluation of the role of IL-13 in IL-12-independent IFN- $\gamma$  production will require the development of neutralizing antibodies to this recently described cytokine.

In summary, these studies have elucidated several novel features elicited during the early periods after invasion by *Leishmania* that may contribute to the survival of this parasite in host macrophages. First, *Leishmania* promastigotes enter macrophages by a pathway that evades the induction of cytokines implicated in orchestrating the cellular immune response, particularly IL-12 (71). Second, a striking burst in IL-4 transcription occurs in the CD4<sup>+</sup> T cell population that peaked at 4 d in all strains of mice analyzed. The simultaneous induction of IL-2, IL-10, IL-13, and IFN- $\gamma$  in most strains suggests that a Th0 population has been stimulated. In contrast to resistant strains, susceptible BALB/c mice fail to downregulate IL-4 transcription, an observation that may underlie the genetic susceptibility of this strain. Finally, amastigotes induce IL-12 from macrophages in a manner similar to other intracellular pathogens (16, 45, 51). Stage-specific evasion of IL-12 induction delays the development of Th1 CD4<sup>+</sup> T cells to allow transformation of the parasite to a developmental form more capable of withstanding the enhanced microbicidal systems of the activated macrophage.



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Address correspondence to Dr. Richard M. Locksley, University of California, San Francisco, 521 Parnassus Avenue, Box 0654, Rm C-443, San Francisco, CA 94143-0654.

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